Novel and Established Potassium Channel Openers Stimulate Hair Growth *In Vitro*: Implications for their Modes of Action in Hair Follicles

Gareth C. Davies,* M. Julie Thornton,* Tracey J. Jenner,* Yi-Ju. Chen,* John B. Hansen,† Richard D. Carr,† and Valerie A. Randall*

*Department of Biomedical Sciences, University of Bradford, Bradford, West Yorkshire, UK; †Novo Nordisk, Discovery Research, Bagsvaerd, Denmark

Although ATP-sensitive potassium (K_{ATP}) channel openers, e.g., minoxidil and diazoxide, can induce hair growth, their mechanisms require clarification. Improved drugs are needed clinically. but the absence of a good bioassay hampers research. K_{ATP} channels from various tissues contain subtypes of the regulatory sulfonylurea receptor, SUR, and pore-forming, K^+ inward rectifier subunits, Kir6.X, giving differing sensitivities to regulators. Therefore, the *in vitro* effects of established potassium channel openers and inhibitors (tolbutamide and glibenclamide), plus a novel, selective Kir6.2/SUR1 opener, NNC 55-0118, were assessed on deer hair follicle growth in serum-free median without streptomycin. Minoxidil (0.1–100 μM, $p<0.001$), NNC 55-0118 (1 mM, $p<0.01$; 0.1, 10, 100 μM, $p<0.001$), and diazoxide (10 μM, $p<0.01$) increased growth. Tolbutamide (1 mM) inhibited growth ($p<0.001$) and abolished the effect of 10 μM minoxidil, diazoxide and NNC 55-0118; glibenclamide (10 μM) had no effect, but prevented stimulation by 10 μM minoxidil. Phenol red stimulated growth ($p<0.001$), but channel modulator responses remained unaltered. Thus, deer follicles offer a practical, ethically advantageous *in vitro* bioassay that reflects clinical responses *in vivo*. The results indicate direct actions of K_{ATP} channel modulators within hair follicles via two types of channels, with SUR 1 and SUR 2, probably SUR2B, sulfonylurea receptors.

Key words: bioassay/diazoxide/glibenclamide/minoxidil/NNC 55-0118/organ culture/phenol red/tolbutamide
J Invest Dermatol 124:686–694, 2005

Minoxidil, diazoxide, and pinacidil are all drugs that stimulate human hair growth, although none were designed to do so (Koblenzer and Baker, 1968; Burton *et al*, 1975; Burton and Marshall, 1979; Goldberg, 1988; Price *et al*, 1999). Minoxidil, currently the main topical treatment for hair loss (Olsen *et al*, 2002; Dawber and Rundegren, 2003), was originally developed as an anti-hypertension therapy. The actual mechanisms by which these pharmaceuticals cause hypertrichosis are uncertain (Messenger and Rundegren, 2004), partly because there is currently no practical, established, reliable bioassay. Although a local vasodilatation action has been suggested, it is not clear whether topical minoxidil actually increases skin blood flow (Wester *et al*, 1984; Bunker and Dowd, 1988) and minoxidil is able to directly stimulate the growth of neonatal mouse vibrissae follicles in organ culture (Buhl *et al*, 1989), suggesting it can act directly on the cells of specialized hair follicles. Since diazoxide, pinacidil, and minoxidil, metabolized to minoxidil sulfate, are all known to open adenosine triphosphate-sensitive potassium (K_{ATP}) channels (Lawson, 1996), they probably stimulate hair growth by this mechanism.

K_{ATP} channels are found in many tissues including the heart, pancreatic β cells, brain, skeletal and smooth muscles, and kidney (Seino and Miki, 2004). Recent research has yielded much more information about the structure and function of these channels. The K_{ATP} channel is constructed as a 4:4 hetero-octamer (see Fig 1); this includes the regulatory sulfonylurea receptor subunits, abbreviated to SUR, and the pore-forming, K^+ inward rectifier subunits, abbreviated to Kir and designated as Kir6.X in the systematic naming of potassium channels, which control the ability of potassium ions to pass through the membrane (Inagaki *et al*, 1995; Clement *et al*, 1997; Seino and Miki, 2004). The presence of both types of subunit is necessary for K^+ channel activity. Currently, two forms of the Kir6.X subunits are known: Kir6.1 and Kir6.2. There are also two isoforms of SUR: SUR1, which combines with Kir6.2 to form the K_{ATP} channels of pancreatic β cells and neurones, etc. (Kir6.2/SUR1), and SUR2, which in two splice variants, SUR2A and SUR2B, combines with the channel protein to form the K_{ATP} channels of cardiac muscle (Kir6.2/SUR2A) and non-vascular smooth muscle (Kir6.2/SUR2B) (Seino and Miki, 2004). In vascular smooth muscle, SUR2B appears to be able to combine with either Kir6.2 or Kir6.1 (Gribble and Reimann, 2003). This range of different K_{ATP} channel forms means that different potassium channel openers and inhibitors, such as the sulfonylureas, tolbutamide, and glibenclamide, used to close channels in pancreatic β cells in diabetics to release insulin, have varying abilities to affect channels in different tissues. Which K_{ATP} channel subtypes are present in the hair

Abbreviations: K_{ATP}, ATP-sensitive potassium; Kir, K^+ inward rectifier subunits; SUR, sulfonylurea receptor

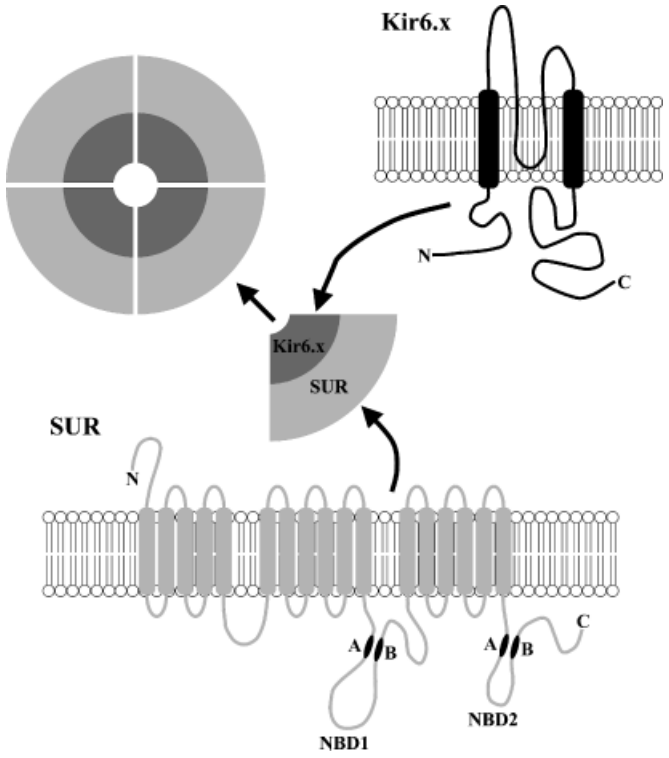

Figure 1
A diagrammatic representation of the structure of ATP-sensitive potassium (K_{ATP}) channels. K_{ATP} channels are composed of two physically associated subunits: the sulfonylurea receptor, SUR and the inward rectifier, Kir6.x in an octameric complex. The central four Kir6.X subunits form the pore through which potassium channels move, whereas the sulfonylurea receptors are regulatory. Different forms of the subunits give different affinities for potassium channel regulators. Note the presence of two intracellular nucleotide binding domains (NBD), consisting of Walker A and B motifs joined by a conserved linker sequence in the SUR protein. Adapted from Bryan and Aguilar-Bryan (1999), Dunne (1999), Miki *et al* (1999), and Fujita and Kurachi (2000).

follicle is currently unclear, although Li *et al* (2001) have detected gene expression of SUR2B receptors in cultured dermal papilla cells. Understanding this could facilitate the development of more selective drugs focused on the particular types of channel present; these could be potentially more powerful in stimulating hair growth or regulating other tissues, such as the pancreas, without producing unwanted hair growth.

Several studies have reported that potassium channel openers affect cultured follicles (Buhl *et al*, 1989, 1992; Harmon *et al*, 1993; Waldon *et al*, 1993; Kamiya *et al*, 1998). But, the range of culture systems used, the different types of follicles studied, the various criteria measured (e.g., follicle growth, cysteine incorporation), and the often conflicting results, make it impossible to determine the exact effect of these drugs on the hair follicle. Nevertheless, a number of common themes emerge. In each study, the lowest concentration(s) investigated had no significant effect on follicle growth, but as the potassium channel opener concentration increased, it appeared to stimulate the follicle, before the response decreased, even becoming inhibitory. The concentrations at which these events occur vary, however, probably because of the different culture systems used. Unfortunately, the stimulatory effects of minoxidil in most of these studies may be due to an attenuation of the inhibitory effects of serum on growth, rather than a direct effect *per se* on follicle growth (Philpott *et al*, 1990). Therefore, the effects of potassium channel openers need to be investigated using the superior serum-free follicle culture system described by Philpott *et al* (1996).

In addition, all previous *in vitro* hair growth studies using potassium channel openers were conducted in the presence of streptomycin, an aminoglycoside antibiotic that can cause hypertrichosis (Sinclair, 2000). Streptomycin has an inhibitory action on both K^+ (Takeuchi and Wangemann, 1993; Gu and Kong, 1997; Rouzaire-Dubois and Dubois, 1998; Murakami *et al*, 1999), and Ca^{2+} channels (Gu and Kong, 1997; Murakami *et al*, 1999), with its action on voltage-dependent K^+ channels even inhibiting the growth of cells in culture (Rouzaire-Dubois and Dubois, 1998). Although a direct effect of streptomycin on K_{ATP} channels has not been demonstrated, an effect on other K^+ channel types may interfere with the putative K_{ATP} channel action of minoxidil in cultured follicles. Indeed, streptomycin did prevent minoxidil from potentiating the mitogenic effects of insulin-like growth factor-1 and other factors on NIH 3T3 fibroblasts (Sanders *et al*, 1996). Similarly, when penicillin and streptomycin were used in the culture medium higher concentrations of minoxidil were needed to stimulate increased growth of human epidermal keratinocytes (Boyera *et al*, 1997). Therefore, it is important that streptomycin is excluded from studies investigating potassium openers to prevent experimental artifacts. A varying response to streptomycin may partially explain the contrasts between the effects of potassium channel openers in different follicle culture systems.

As a result of all these factors a new consistent, practical *in vitro* bioassay is needed to facilitate the development of more effective hair growth promoters or to assess the potential risk of unwanted hair growth side effects. Although human hair follicles are the ideal tissue, the small numbers of follicles available limits their use in a bioassay. Laboratory rodents are also impractical as the pelage follicles are too small; whisker follicles are possible but these are unusual follicles with large venous sinuses, specialized as neuroreceptors, which may well have different responses (Randall *et al*, 2003). The red deer (*Cervus elaphus*) is an attractive animal to use for hair growth studies. It has large, easy to study follicles whose seasonal growth cycles mean follicles in any given area are at the same stage of the hair cycle. In addition, each year the stag grows an androgen-dependent mane in the breeding season (Lincoln and Kay, 1971) also enabling studies on androgen-dependent follicles *in vitro*. For a bioassay, the large number of follicles in identical hair cycle stage available from red deer is a major advantage; this allows a variety of experimental conditions to be compared within the same individual animal. This model also has ethical advantages, as skin is available from animals bred and harvested for food. The culture of red deer follicles is well established in our laboratory (Thomas *et al*, 1994; Thornton *et al*, 1994, 1996). The morphology and pattern of cell division in cultured deer follicles resemble that observed *in vivo*, and their growth rate is comparable with the rate of coat growth *in vivo*. The seasonal hormonal response of red deer follicles *in vivo* is also retained in culture (Thomas *et al*, 1994; Thornton *et al*, 1996).

Therefore, isolated red deer anagen follicles cultured in serum-free media in the absence of streptomycin were investigated to examine their potential as a bioassay for the assessment of hair growth-promoting drugs and to elucidate further the mode of action of potassium channel openers on hair growth. The effects of the established potassium channel openers, minoxidil and diazoxide, and a new drug, NNC 55-0118 (NovoNordisk, Bagsvaed, Denmark) (Nielsen *et al*, 2002; Dabrowski *et al*, 2003; Jansson *et al*, 2003), on the rate of follicular growth were compared. NNC 55-0118 only opens Kir6.2/SUR1channels (Nielsen *et al*, 2002; Dabrowski *et al*, 2003). Although minoxidil must be metabolized into minoxidil sulfate to have its effects (Hamamoto and Mori, 1989; Buhl *et al*, 1990; Dooley *et al*, 1991; Anderson *et al*, 1998), minoxidil sulfate was not used in the assays because it is unstable in aqueous solution, undergoing rapid hydrolysis (Johnson *et al*, 1982; Harmon *et al*, 1993). Follicles are expected to metabolize minoxidil to minoxidil sulfate by a sulfotransferase enzyme predominantly located in the lower outer root sheath in vibrissae follicles (Hamamoto and Mori, 1989; Buhl *et al*, 1990; Dooley *et al*, 1991).

To help clarify whether these potassium channel openers were acting via K_{ATP} channels in the follicle, the response of cultured follicles to K_{ATP} channel inhibitors, the sulfonylureas, tolbutamide, and glibenclamide, were also assessed. Tolbutamide selectively inhibits Kir6.2/SUR1 K_{ATP} channels with low potency, whereas glibenclamide non-selectively blocks both SUR1 and SUR2B forms with high affinity (Babenko *et al*, 1998; Ashcroft and Gribble, 2000; Fujita and Kurachi, 2000; Gribble and Reimann, 2003; Seino and Miki, 2004).

The pH indicator, phenol red (phenolsulfonphthalein), in the culture media may also interfere with agents that modulate potassium channels (Messenger and Birch, personal communication). The phenol red used to prepare culture media commonly contains a number of lipophilic impurities generated during its synthesis (Kym *et al*, 1996), the proportion of which varies between batch and supplier. Therefore, the effects of a commercial phenol red preparation on follicle growth and response to potassium channel modulators were also investigated.

Results

Potassium channel openers stimulated follicle growth in normal growth media Red deer hair follicles grew under all experimental conditions for 8 d. An example of the growth of an isolated follicle is shown in Fig 2. No difference in gross morphology was observed between experimental groups. All concentrations of minoxidil (0.1–100 μM, $p<0.001$) increased follicle growth over the culture period (Fig 3*a*). Diazoxide at 10 μM significantly stimulated follicle growth ($p<0.01$), but at the other concentrations had no significant effect (0.1 μM, $p=0.598$; 1 μM, $p=0.952$; 100 μM, $p=0.952$; Fig 3*b*). All concentrations of NNC 55-0118 significantly increased growth over the culture period (1 μM: $p<0.01$; 0.1, 10, 100 μM $p<0.001$ Fig 3*c*).

Tolbutamide inhibited follicle growth in normal growth media Incubation of the isolated follicles with 1 mM tolbutamide significantly inhibited the stimulatory effects of 10 μM minoxidil ($p<0.05$; Fig 4*a*), diazoxide ($p<0.001$; Fig 4*b*), and NNC 55-0118 ($p<0.001$; Fig 4*c*). In the minoxidil and diazoxide, but not the NNC 55-0118, experiments, however, follicles incubated with tolbutamide alone grew at a significantly lower rate than the vehicle (minoxidil study: $p<0.05$; diazoxide study: $p<0.01$; NNC 55-0118 study: $p=0.488$). This was, therefore, investigated more fully and confirmed with a larger sample number ($p<0.001$; Fig 5*a*).

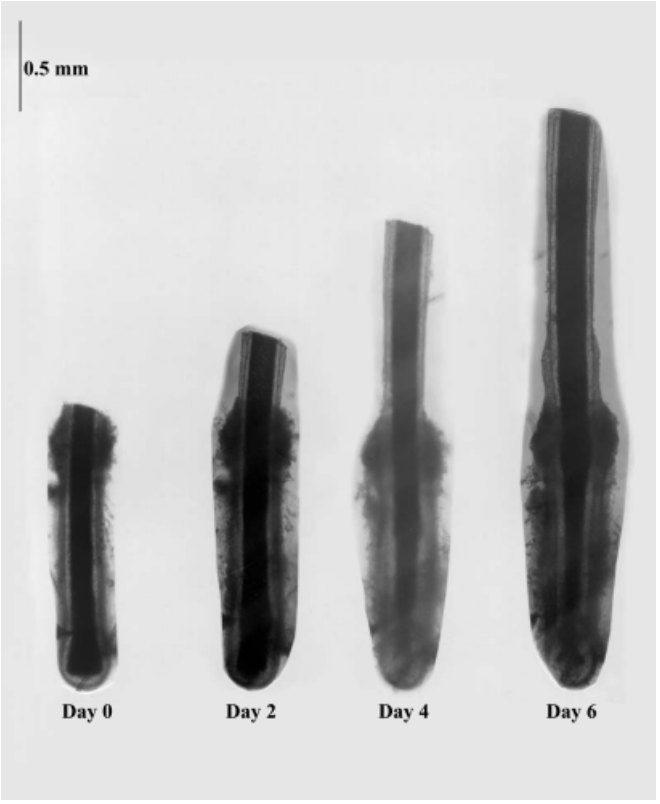

Figure 2
Sequential photographs of a red deer hair follicle showing new growth *in vitro*. Light micrographs taken under an inverted microscope without phase contrast showing the initial length and sequential growth of a hair follicle isolated from the red deer mane. *Bar* = 0.5 mm.

Effects of potassium channel regulators in phenol red-free media Follicles maintained in phenol red-free media grew at a significantly ($p<0.001$) lower rate than when the media were supplemented with 10 μg per mL phenol red, the concentration normally used in the formulation of William's E medium (Fig 6). In phenol red-free conditions, tolbutamide (1 mM) significantly ($p<0.001$) reduced (Fig 5*b*), and minoxidil (10 μM) significantly ($p<0.001$) increased the rate of follicle growth (Fig 7). A combination of minoxidil and tolbutamide resulted in an inhibition of minoxidil-stimulated growth ($p<0.001$; Fig 8). Glibenclamide (10 μM) treatment did not alter the growth of follicles compared with control ($p=0.991$; Fig 9), but did completely abolish ($p<0.001$) the stimulatory effects of minoxidil (Fig 10). Interestingly, follicles incubated with a combination of minoxidil and glibenclamide also grew at a significantly lower rate then follicles incubated with the vehicle ($p<0.001$) or glibenclamide alone ($p<0.05$).

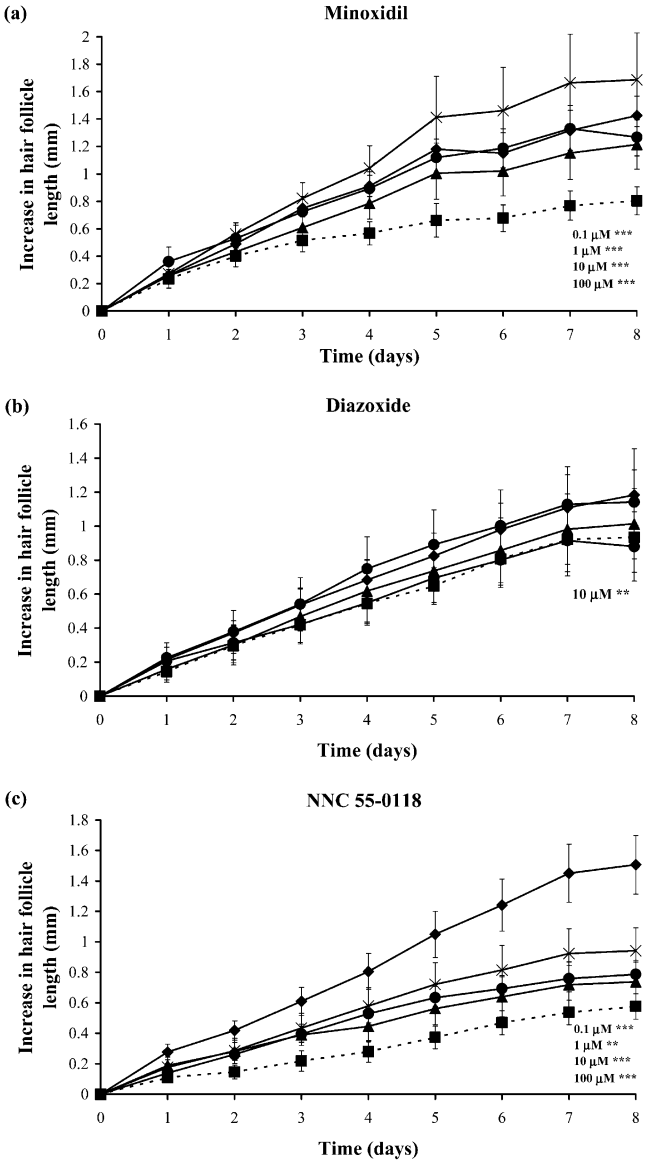

Figure 3
Potassium channel openers stimulate red deer hair follicle growth *in vitro*. Anagen hair follicles isolated from the mane of the red deer winter coat were incubated in the presence of minoxidil (*a*), diazoxide (*b*), or NNC 55-0118 (*c*) at concentrations of 0 (containing the vehicle 0.01% DMSO; ■), 0.1 (●), 1 (▲), 10 (◆) 100 μM (×) n = 6 animals for each). The increase in follicle length was measured sequentially with an inverted microscope fitted with an eyepiece measuring graticle over 8 d. Values are mean ± SEM of n = 5 animals; a minimum of six follicles were isolated per animal and the mean was calculated to represent each animal. Comparison between means of the animal means over the entire culture period was performed as described in the Materials and Methods; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

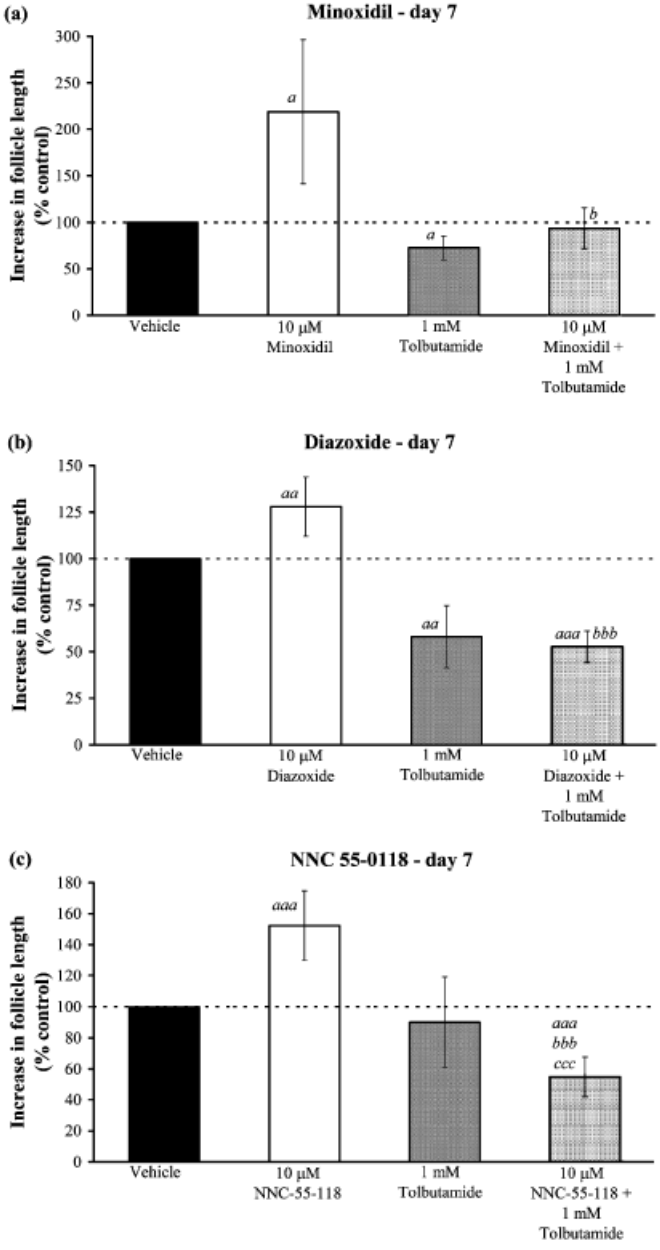

Figure 4
The hair follicle growth-promoting effects of minoxidil, diazoxide, and NNC 55-0118 are inhibited by tolbutamide. Anagen hair follicles isolated from the mane of the red deer winter coat were maintained with the vehicle (0.01% DMSO), 10 μM minoxidil, 10 μM diazoxide, 10 μM NNC 55-0118, 1 mM tolbutamide, 10 μM of minoxidil and 1 mM tolbutamide, 10 μM of diazoxide and 1 mM tolbutamide, or 10 μM of NNC 55-0118 and 1 mM tolbutamide. The increase in follicle length after 7 d culture is expressed as a percentage of control follicle growth. Values are mean ± SEM of n = 6 animals for the minoxidil experiment (*a*), n = 5 for the diazoxide experiment (*b*), or n = 6 for the NNC 55-0118 experiment (*c*). Comparison between means over the entire culture period were performed on the original data as described in Materials and Methods; *a*, $p<0.05$; *aa*, $p<0.01$; *aaa*, $p<0.001$ compared with control; *b*, $p<0.05$; *bbb*, $p<0.001$ compared with potassium channel opener; *ccc*, $p<0.001$ compared with tolbutamide.

Discussion

Deer hair follicles grew readily and maintained an anagen morphology *in vitro* in the absence of serum and streptomycin (Fig 2). The increase in the rate of follicle growth with minoxidil, diazoxide, or NNC 55-0118 treatment (Fig 3) demonstrates that red deer follicles have the capacity to respond to various potassium channel openers directly. This *in vitro* effect confirms previous reports that potassium channel openers are capable of stimulating hair growth independent of any vascular effects (Buhl *et al*, 1989, 1992) in the absence of any potential interference from serum or streptomycin. The *in vitro* increase in follicle growth caused by minoxidil is analogous to the *in vivo* increase in follicular DNA synthesis in the bald scalp of stump-tailed macaques

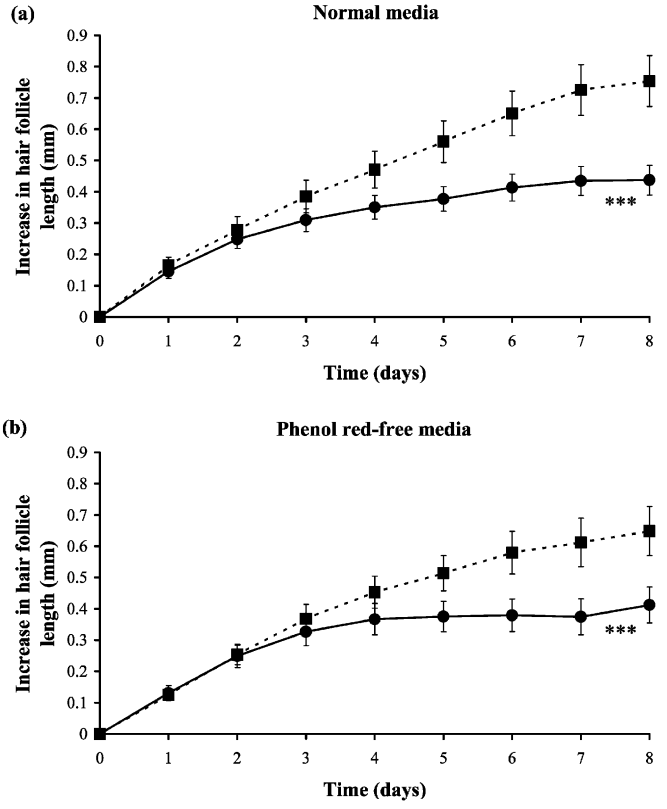

Figure 5
Tolbutamide inhibits red deer hair growth *in vitro*. Anagen hair follicles isolated from the mane of the red deer winter coat were incubated in the presence of either the vehicle (0.01% DMSO) (■) or 1 mM tolbutamide (●). The effects of tolbutamide on follicle growth in the presence (*a*; n = 23 animals) or absence (*b*; n = 14 animals) of 10 μg per mL phenol red were compared. Values are mean ± SEM; ***p < 0.001.

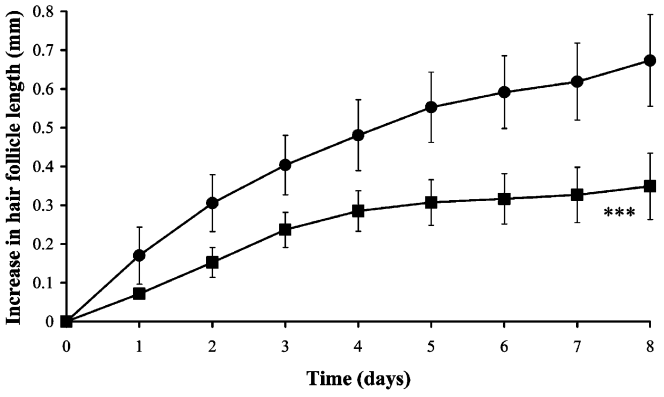

Figure 6
Phenol red stimulates red deer hair follicle growth *in vitro*. Anagen hair follicles isolated from the mane of the red deer winter coat were incubated in phenol red-free William's E medium supplemented with 5 mM glucose, 0.01% DMSO (■) and 10 μg per mL phenol red (●). Values are mean ± SEM of n = 6 animals; ***p < 0.001.

(Uno *et al*, 1987), the higher incidence of mitotic figures in the hair follicles of CD1 mice (Schop and Goldberg, 1988), and the increase in proliferation index of hairs plucked from the frontal scalps of bald men (Kiesewetter *et al*, 1991) following topical minoxidil treatment.

The increased growth stimulated by minoxidil in red deer hair follicles in serum-free media parallels the increased

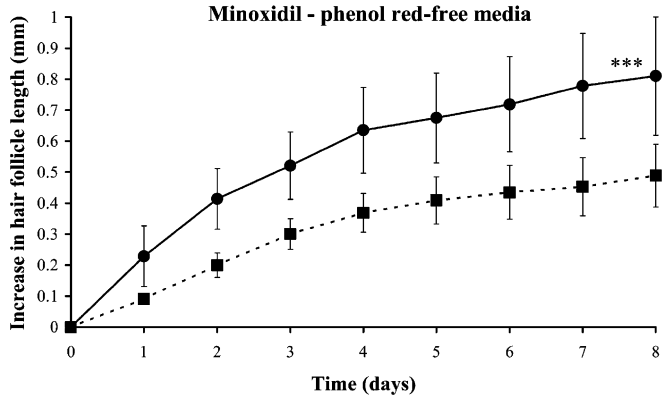

Figure 7
Minoxidil stimulates red deer hair growth in phenol red-free media. Anagen hair follicles isolated from the mane of the red deer winter coat were incubated in phenol red-free media with either the vehicle (0.01% DMSO) (■) or 10 μM minoxidil (●). Values are mean ± SEM of n = 8 animals; ***p < 0.001.

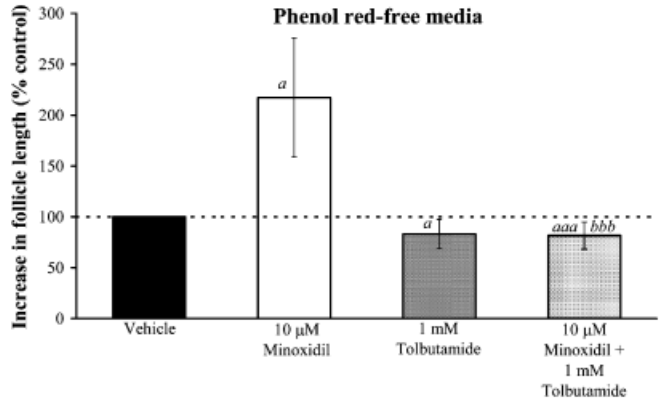

Figure 8
The hair follicle growth-promoting effect of minoxidil is inhibited by tolbutamide in phenol red-free media. Anagen hair follicles isolated from the mane of the red deer winter coat were maintained with either the vehicle (0.01% DMSO), 10 μM minoxidil, 1 mM tolbutamide, or 10 μM of minoxidil and 1 mM tolbutamide. The increase in follicle length after 7 d culture is expressed as a percentage of control follicle growth. Values are mean ± SEM of n = 6 animals; *a*, p < 0.05, *aaa*, p < 0.001 compared with control; *bbb*, p < 0.001 compared with minoxidil.

DNA synthesis by human follicles in serum-free media (Imai *et al*, 1993). The stronger response to minoxidil than diazoxide by deer follicles also reflects human hair growth *in vivo*, since hypertrichosis in adults treated with oral minoxidil is much more frequent than with oral diazoxide (Koblenzer and Baker, 1968; Burton and Marshall, 1979; Zins, 1988). Topical minoxidil also appears to be slightly more effective than diazoxide (Olsen *et al*, 1985; Olsen and Weiner, 1987; Roenigk, 1988). Therefore, cultured red deer follicles grown in the absence of serum and streptomycin appear to be a relevant, practical, ethically advantageous model for studying potassium channel openers, and probably other novel hair growth-promoting therapeutics.

The stimulatory effect of a commercial phenol red preparation on follicle growth in this system (Fig 6) is an interesting finding. Impurities from the synthesis of phenol red are probably responsible. Reported impurities include those with an estrogenic effect (Berthois *et al*, 1986; Bindal and

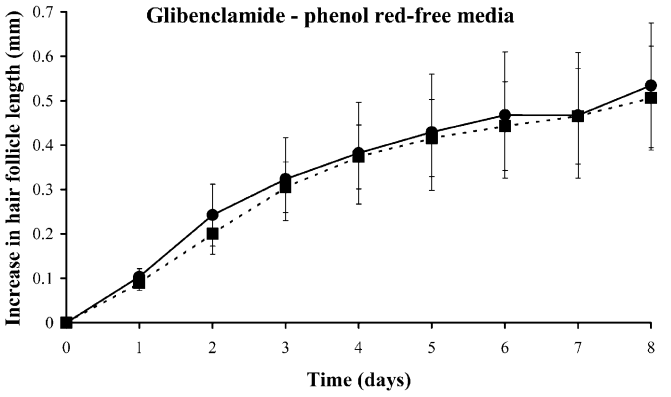

Figure 9
Glibenclamide does not affect the growth of red deer hair follicles *in vitro* in phenol red free media. Anagen hair follicles isolated from the mane of the red deer winter coat were incubated in phenol red-free media with either the vehicle (0.01% DMSO) (■) or 10 μM glibenclamide (●). Values are mean ± SEM of n = 7 animals.

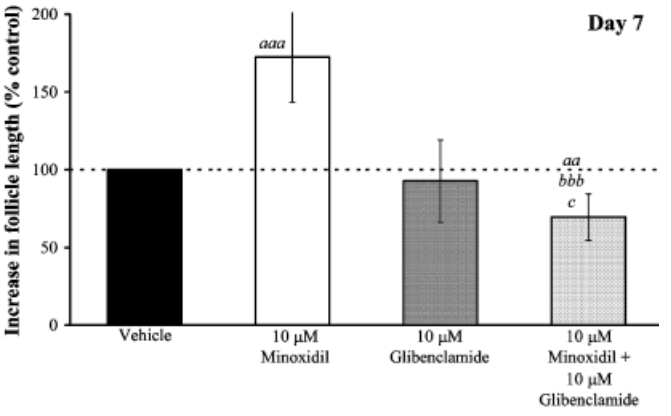

Figure 10
The hair growing effect of minoxidil is inhibited by glibenclamide in phenol red-free media. Anagen hair follicles isolated from the mane of the red deer winter coat were maintained in phenol red-free William's E medium supplemented with 5 mM glucose and either the the vehicle (0.01% DMSO), 10 μM minoxidil (ν = 5) glibenclamide, or 10 μM of NNC 55-0118 and 10 μM glibenclamide. Values are mean ± SEM of n = 7 animals; *aa*, $p < 0.01$; *aaa*, $p < 0.001$ compared with control; *bbb*, $p < 0.001$ compared with minoxidil; *c*, $p < 0.05$ compared with glibenclamide.

Katzenellenbogen, 1988), a cytotoxic activity (Grady *et al*, 1991; Kym *et al*, 1996), and an effect on cellular Na^+ and K^+ homeostasis (Hopp and Bunker, 1993; Lubin, 1993; Kym *et al*, 1996). Although the presence of phenol red did not alter the follicular response to potassium channel modulators, it may obscure the actions of other agents capable of affecting hair growth. It seems preferable to carry out assessment of hair growth-promoting drugs in phenol red-free media in future.

The potassium channel inhibitor, tolbutamide (1 mM), antagonized the effects of all three potassium channel openers, minoxidil, diazoxide, or NNC 55-0118 at 10 μM (Fig 4), strongly supporting the concept that the potassium channel openers were acting directly on K_{ATP} channels. Both tolbutamide (Fig 8) and another potassium channel inhibitor, glibenclamide (10 μM) (Fig 10), completely abolished the stimulatory effects of minoxidil (10 μM) in phenol red-free media. This contrasts with a previous report where tolbutamide (5–500 μM) or glibenclamide (0.5–50 μM) had no effect on minoxidil (1 mM)-induced ^{35}S-cysteine incorporation into neonatal mouse vibrissae follicles (Buhl *et al*, 1993). Although these differences may indicate variations in the action of minoxidil (Buhl *et al*, 1990) between adult deer follicles and the neonatal, specialized mouse vibrissae follicles, the effects are more likely to be due to methodological differences between the studies. Most importantly, control follicles lost morphology and underwent necrosis in the serum-containing mouse vibrissae culture system; this did not occur in the serum–free deer follicle culture. In addition, streptomycin was included in the vibrissae study and only increased cysteine incorporation was measured, which may not equate to greater hair length. Finally, the concentration of the drugs used differed; although the glibenclamide concentrations are similar, Buhl *et al* (1993) used a minoxidil concentration 100-fold higher and lower tolbutamide concentrations.

Tolbutamide alone (1 mM) also inhibited follicle growth in culture both in the presence and absence of phenol red (Fig 5). This suggests that open K_{ATP} channels may be necessary for normal hair follicle growth, at least in culture. Glibenclamide (10 μM), however, did not affect the rate of follicle growth compared with the vehicle (Fig 9). This may reflect differences in sensitivity of channels present in the follicle to the two sulfonylureas. Alternative possibilities could be a non-specific toxic effect of the tolbutamide on the hair follicles or action by another mechanism apart from via potassium channel effects. A toxic effect seems unlikely as follicles continued to grow throughout the experiment, the growth of tolbutamide follicles was normally distributed (e.g., after 7 d of culture Kolmogorov–Smirnov $Z = 0.518$, $p = 0.951$), and their morphology was normal. In addition, growth was also inhibited by tolbutamide at 0.1 mm (data not shown). Although action by another mechanism cannot be excluded, tolbutamide at this concentration inhibits native (Trube *et al*, 1986; Ashcroft *et al*, 1989; Valdeolmillos *et al*, 1992) and recombinant (Gribble *et al*, 1997b) K_{ATP} channels.

The stimulation of hair follicle growth *in vitro* by various potassium channel openers and its opposition by potassium channel inhibitors strongly support a direct role in hair follicles for potassium channel regulators rather than an action mediated purely via the follicle vasculature. The effects of this range of regulators, however, suggest that the follicle mechanism is not simple. Hair follicles are complex organs involving several different tissue types *in vivo* including capillaries and nerves; even *in vitro*, where any possible vascular effects can be eliminated, follicles include the regulatory mesenchyme-derived dermal papilla cells, the epithelial cells that actively divide to enable the hair to grow, the melanocytes that produce the hair pigment and the dermal or connective tissue sheath that surrounds the follicle, isolating it from the dermis. Both human epidermal keratinocytes (Boyera *et al*, 1997) and follicular dermal papilla cells (Lachgar *et al*, 1998) respond to potassium channel regulators *in vitro*, suggesting that both cell types may contain potassium channels. Although Nakaya *et al* (1994) were unable to demonstrate K_{ATP} channels in either cultured dermal papilla cells or outer root sheath cells using the patch clamp method to examine conductance through the membrane, Li *et al* (2001) were able to detect gene expres-

sion of SUR2B receptors in dermal papilla cells. They have suggested that cultured dermal papilla cells respond to minoxidil via a complex mechanism involving SUR2B receptors and adenosine A1 and A2 receptors.

In the experiments reported here, the greater stimulatory effect of minoxidil on hair growth compared with diazoxide suggests the involvement of a SUR2-based channel. Although diazoxide activates both the Kir6.2/SUR1 (e.g., pancreas) and the Kir6.2/SUR2B (e.g., vascular) channels, with minor effects on the Kir6.2/SUR2A channels (e.g., cardiac muscle), minoxidil has no significant effects on the Kir6.2/SUR1 K_{ATP} channel, suggesting that their hair growth-promoting effects will operate via a SUR2 form (Ashcroft and Gribble, 2000; Seino and Miki, 2004). Indeed, minoxidil sulfate exhibits a biphasic affinity for Kir6.2/SUR2B channels (Schwanstecher *et al*, 1998). On the other hand, the growth stimulation elicited by the novel, channel-specific, potassium channel opener, NNC 55-0118 (Nielsen *et al*, 2002; Dabrowski *et al*, 2003; Jansson *et al*, 2003), indicates an effect mediated through SUR1/Kir6.2 potassium channels. The inhibitory effects of tolbutamide both alone, and opposing the action of NNC 55-0118, also supports an action through SUR1/Kir6.2 potassium channels as tolbutamide has a high affinity for the SUR1 receptor. Both tolbutamide and glibenclamide, however, also inhibited minoxidil stimulation, suggesting an action through a SUR2B form as glibenclamide acts on SUR2B with high affinity and tolbutamide with low affinity (Babenko *et al*, 1998; Ashcroft and Gribble, 2000; Fujita and Kurachi, 2000; Gribble and Reimann, 2003; Seino and Miki, 2004). Overall, this would suggest that both SUR1/Kir6.2- and SUR2B/Kir6.2-based K_{ATP} channels are involved in hair follicle growth. This parallels a recent report that pig urethra contains both SUR1 and SUR2B potassium channels (Yunoki *et al*, 2003). Based on all current knowledge, it seems feasible to hypothesize that potassium channel regulators acting via SUR 2B may influence hair growth via the dermal papilla, whereas those acting via SUR1 receptors such as NNC 55-0118 may act directly on the keratinocytes of the follicle. A role for SUR2 forms in keratinocytes cannot be excluded at the moment. Since the characterization of K_{ATP} channels is not yet complete, further advances in our understanding of these channels in other tissues may yield greater clarification of their mechanisms in the hair follicle. Particularly interesting currently are the additional K_{ATP} channels recently detected in the mitochondria of guinea pig heart myocytes (Sato *et al*, 2004). These have different characteristics from the established ones in the sarcolemma of the cells and, although both channels are activated by minoxidil, the mitochondrial forms are much more sensitive to it.

In conclusion, the red deer hair follicle culture system provides a useful model for investigating the action of potassium channel openers in the absence of serum and streptomycin. Although caution always has to be exercised when extrapolating from animal models to human tissues, the relative response of minoxidil and diazoxide in this system reflects their clinical effects in adult human beings. The availability of large numbers of follicles from a single animal without many of the normal ethical drawbacks involved in animal work means that this culture system offers a practical *in vitro* bioassay, which should allow a more complete investigation into the mechanism of action of potassium channel openers and other hair growth-promoting therapeutics in the hair follicle. The *in vitro* stimulation of hair growth by potassium channel openers, and its inhibition by channel inhibitors, is consistent with a direct action of potassium channel regulators on potassium channels in the hair follicle. The effects of a range of regulators that act by different SURs on potassium channels suggest that the hair follicle contains at least two types of potassium channels with SUR1 and SUR2 receptors. Further studies may facilitate the development of more selective and more potent hair-promoting treatments.

Materials and Methods

Materials William's E medium, L-glutamine, and amphotericin B, were supplied by Gibco BRL (Romford, UK). phenol red-free William's E medium, phenol red solution, minoxidil, diazoxide, D-glucose, and penicillin were supplied by Sigma (Poole, UK). NNC 55-118 (6-chloro-3-isopropylamino-4H-thieno[3,2-*e*]-1,2,4-thiadiazine 1,1-dioxide) was a kind gift from Novo Nordisk A/S (Bagsvćrd, Denmark). Tolbutamide and glibenclamide were purchased from ICN (Basingstoke, UK). Tissue culture plasticware and other laboratory consumables were obtained from SLS (Nottingham, UK).

Animals This study was conducted with skin samples which were by-products, from mature red deer stags which had been harvested for food. All animals used in the study were bred in Ireland or the UK (longitude: 0–10°W; latitude: 51–57°N), before being transferred to a farm (longitude: 1°30′W; latitude: 53°30′N) for a minimum of 2 wk before harvesting. The stags were killed by the approved method of pithing and exsanguination during the growth of the winter (breeding season) coat (September, October, and November; age: 16–20 months; weight: 90–110 kg). Samples were collected into serum-free media at 4 °C and transported to the laboratory on ice.

Isolation and maintenance of anagen hair follicles Anagen hair follicles were isolated by microdissection from red deer mane skin. Each hair follicle was then washed in PBS, before being transferred to an individual well of a 24-well plate containing 1 mL William's E medium, supplemented with 2 mM L-glutamine, 100 IU per mL penicillin, 2.5 μg per mL amphotericin B, and 5 mM D-glucose (final media concentration = 16.1 mM). For each stag used, a minimum of six follicles were randomly assigned to each of the different experimental groups and supplemented as described in Results; i.e. if $n = 6$ animals per treatment at least 36 follicles were isolated and cultured in each medium. The follicles were then maintained free-floating at 37°C in an atmosphere of 5% CO_2 and 95% air. The culture medium was changed every 2–3 d throughout the course of the experiment.

Measurement of follicle growth Follicle length was measured every 24 h over an 8-d culture period using a Leitz Labovert inverted microscope (Labovert, Leitz, Germany) with an eyepiece measuring graticle at × 40 magnification. The accuracy of this equipment was ± 0.01 mm. Hair follicle growth (elongation) was defined as the increase in the length of the hair follicle with time in culture (Philpott *et al*, 1990). Follicles that failed to elongate by 0.01 mm/24 h were classed as unviable and excluded from data analysis. The mean growth of isolated follicles per stag for each treatment group was determined prior to calculation of the sample mean. The growth of hair follicles in each experimental group was expressed as the mean increase in length (mm) ± SEM of the number of animals used. The effect of the different treatments with time in culture was analyzed by two-factor, within-subjects ANOVA using the SPSS statistical analysis program (SPSS Inc., Chicago, Illinois). If the sample means of the different experimental groups differed significantly ($p < 0.05$), selected experimental group means were compared using a Student's paired *t* test with Sidak's correction for

multiple comparisons. Follicle growth was also graphically expressed as a percentage of the control follicle growth at day 7.

This research was partially supported by a University of Bradford Research Studentship and partially by Novo Nordisk, Discovery Research, Denmark.

DOI: 10.1111/j.0022-202X.2005.23643.x

Manuscript received July 23, 2004; revised October 26, 2004; accepted for publication November 15, 2004

Address correspondence to: Professor Valerie A. Randall, Department of Biomedical Sciences, University of Bradford, Bradford, West Yorkshire, BD7 1DP, UK. Email: v.a.randall@bradford.ac.uk

References

Anderson RJ, Kudlacek PE, Clemens DL: Sulfation of minoxidil by multiple human cytosolic sulfotransferases. Chem Biol Interact 109:53–67, 1998

Ashcroft FM, Gribble FM: Tissue-specific effects of sulfonylures: Lessons from studies of cloned KATP channels. J Diabetes Complicat 14:192–196, 2000

Ashcroft FM, Kakei M, Gibson JS, Gray DW, Sutton R: The ATP- and tolbutamide-sensitivity of the ATP-sensitive K-channel from human pancreatic B cells. Diabetologia 32:591–598, 1989

Babenko AP, Aguilar-Bryan L, Bryan J: A view of Sur/KIR6.X, K_{ATP} channels. Annu Rev Physiol 60:667–687, 1998

Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS: Phenol red in tissue culture media is a weak estrogen: Implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci USA 83:2496–2500, 1986

Bindal RD, Katzenellenbogen JA: Bis(4-hydroxyphenyl)-[2-(phenoxysulfonyl)-phenyl]methane: Isolation and structure elucidation of a novel estrogen from commercial preparations of phenol red (phenolsulfonphthalein). J Med Chem 31:1978–1983, 1988

Boyera N, Galey I, Bernard BA: Biphasic effects of minoxidil on the proliferation and differentiation of normal human keratinocytes. Skin Pharmacol 10:206–220, 1997

Bryan J, Aguilar-Bryan L: Sulfonylurea receptors: ABC transporters that regulate ATP-sensitive K(+) channels. Biochim Biophys Acta 1461:285–303, 1999

Buhl AE, Conrad SJ, Waldon DJ, Brunden MN: Potassium channel conductance as a control mechanism in hair follicles. J Invest Dermatol 101:148S–152S, 1993

Buhl AE, Waldon DJ, Baker CA, Johnson GA: Minoxidil sulfate is the active metabolite that stimulates hair follicles. J Invest Dermatol 95:553–557, 1990

Buhl AE, Waldon DJ, Conrad SJ, *et al*: Potassium channel conductance: A mechanism affecting hair growth both *in vitro* and *in vivo*. J Invest Dermatol 98:315–319, 1992

Buhl AE, Waldon DJ, Kawabe TT, Holland JM: Minoxidil stimulates mouse vibrissae follicles in organ culture. J Invest Dermatol 92:315–320, 1989

Bunker CB, Dowd PM: Alterations in scalp blood flow after epicutaneous application of 3% minoxidil. Br J Dermatol 117:668, 1988

Burton JL, Marshall A: Hypertrichosis due to minoxidil. Br J Dermatol 101: 593–595, 1979

Burton JL, Schutt WH, Caldwell IW: Hypertrichosis due to diazoxide. Br J Dermatol 93:707–711, 1975

Clement JP, Kunjilwar K, Gonzalez G, Schwanstecher M, Panten U, Aguilar-Bryan L, Bryan J: Association and stoichiometry of K(ATP) channel subunits. Neuron 18:827–838, 1997

Dabrowski M, Larsen T, Ashcroft FM, Bondo Hansen J, Wahl P: Potent and selective activation of the pancreatic β-cell type KATP channel by two novel diazoxide analogues. Diabetologia 46:1375–82, 2003

Dawber RP, Rundegren J: Hypertrichosis in females applying minoxidil topical solution and in normal controls. J Eur Acad Dermatol Venereol 17: 271–275, 2003

Dooley TP, Walker CJ, Hirshey SJ, Falany CN, Diani AR: Localization of minoxidil sulfotransferase in rat liver and the outer root sheath of anagen pelage and vibrissa follicles. J Invest Dermatol 96:65–70, 1991

Dunne MJ: Potassium channels, sulphonylurea receptors and control of insulin release. Trends Endocrin Met 10:146–152, 1999

Fujita A, Kurachi Y: Molecular aspects of ATP-sensitive K^+ channels in the cardiovascular system and K^+ channel openers. Pharmacol Therapeut 85: 39–53, 2000

Goldberg MR: Clinical pharmacology of pinacidil, a prototype for drugs that affect potassium channels. J Cardiovasc Pharmacol 12:S41–S47, 1988

Grady LH, Nonneman DJ, Rottinghaus GE, Welshons WV: pH-dependent cytoxicity of contaminants of phenol red for MCF-7 breast cancer cells. Endocrinology 129:3321–3330, 1991

Gribble FM, Reimann F: Differential selectivity of insulin secretagogues. Mechanisms, clinical implications, and drug interactions. J Diabetes Complicat 17:11–17, 2003

Gribble FM, Tucker SJ, Ashcroft FM: The interaction of nucleotides with the tolbutamide block of cloned ATP-sensitive K^+ channel currents expressed in Xenopus oocytes: A reinterpretation. J Physiol 504:35–45, 1997b

Gu F, Kong W: Effects of streptomycin on ionic currents in isolated outer hair cells of guinea pig cochlea. Lin Chuang Er Bi Yan Hou Ke Za Zhi 11:195–197, 1997

Hamamoto T, Mori Y: Sulfation of minoxidil in keratinocytes and hair follicles. Res Commun Chem Pathol Pharmacol 66:33–44, 1989

Harmon CS, Lutz D, Ducote J: Potassium channel openers stimulate DNA synthesis in mouse epidermal keratinocyte and whole hair follicle cultures. Skin Pharmacol 6:170–178, 1993

Hopp L, Bunker CH: Lipophilic impurity of phenol red is a potent cation transport modulator. J Cell Physiol 157:594–602, 1993

Imai R, Jindo T, Miura Y, Mochida K, Takamori K, Ogawa H: Organ culture of human hair follicles in serum-free medium. Arch Dermatol Res 284: 466–471, 1993

Inagaki N, Gonoi T, Clement JP, *et al*: Reconstitution of IKATP: An inward rectifier subunit plus the sulfonylurea receptor. Science 270:1166–1170, 1995

Jansson L, Kullin M, Karlsson FA, Bodin B, Hansen JB, Sanders S: $K(_{ATP})$ channels and pancreatic islet blood flow in anaesthetized rats: Increased blood flow induced by potassium channel openers. Diabetes 52:2043–2048, 2003

Johnson GA, Barsuhn KJ, McCall JM: Sulfation of minoxidil by liver sulfotransferase. Biochem Pharmacol 31:2949–2954, 1982

Kamiya T, Shirai A, Kawashima S, Sato S, Tamaoki T: Hair follicle elongation in organ culture of skin from newborn and adult mice. J Dermatol Sci 17: 54–60, 1998

Kiesewetter F, Langer P, Schell H: Minoxidil stimulates mouse vibrissae follicles in organ culture [letter; comment]. J Invest Dermatol 96:295–296, 1991

Koblenzer PJ, Baker L: Hypertrichosis Lauginosa associated with diazoxide therapy in prepubertal children: A clinicopathologic study. Ann N Y Acad Sci 150:379–382, 1968

Kym PR, Hummert KL, Nilsson AG, Lubin M, Katzenellenbogen JA: Bisphenolic compounds that enhance cell cation transport are found in commercial phenol red. J Med Chem 39:4897–4904, 1996

Lachgar S, Charveron M, Gall Y, Bonafe JL: Minoxidil upregulates the expression of vascular endothelial growth factor in human hair dermal papilla cells. Br J Dermatol 138:407–411, 1998

Lawson K: Potassium channel activation: A potential therapeutic approach? Pharmacol Therapeut 70:39–63, 1996

Li M, Marubayashi A, Nakaya Y, Fukui K, Arase S: Minoxidil induced hair growth is mediated by adenosine in cultured dermal papilla cells: Possible involvement of sulfonylurea receptor 2B as a target of minoxidil. J Invest Dermatol 117:1594–1600, 2001

Lincoln GA, Kay RNB: The seasonal reproductive changes in the red deer stag. J Zool (London) 163:105–123, 1971

Lubin M: An impurity in phenol red opens an ion channel in cultured human cells. *In Vitro* Cell Dev Biol Anim 29A:597–600, 1993

Messenger AG, Rundegren J: Minoxidil: Mechanisms of action on hair growth. Br J Dermatol 150:186–94, 2004

Miki T, Nagashima K, Seino S: The structure and function of the ATP-sensitive K^+ channel in insulin-secreting pancreatic beta-cells. J Mol Endocrinol 22:113–123, 1999

Murakami M, Tokutomi N, Tokutomi Y, Tomita K, Nishi K: Alkalinization-induced K^+ current of the mouse megakaryocyte. Jpn J Pharmacol 79:343–350, 1999

Nakaya Y, Hamaoka H, Kato S, Arase S: Effect of minoxidil sulfate and pinacidil on single potassium channel current in cultured human outer root sheath cells and dermal papilla cells. J Dermatol Sci 7 (Suppl):S104–S108, 1994

Nielsen FE, Bodvarsdottir TB, Worsaae A, *et al*: 6-Chloro-3-alkylamino-4h-thieno[3,2-e]-1,2,4-thiadiazine 1,1- dioxide derivatives potently and selectively activate ATP sensitive potassium channels of pancreatic beta-cells. J Med Chem 45:4171–4187, 2002

Olsen EA, Dunlap FE, Funicella T, *et al*: A randomized clinical trial of 5% topical minoxidil versus 2% topical minoxidil and placebo in the treatment of androgenetic alopecia in men. J Am Acad Dermatol 47:377–385, 2002

Olsen EA, Weiner MS: Topical minoxidil in male pattern baldness: Effects of discontinuation of treatment. J Am Acad Dermatol 17:97–101, 1987

Olsen EA, Weiner MS, Delong ER, Pinnell SR: Topical minoxidil in early male pattern baldness. J Am Acad Dermatol 13:185–192, 1985

Philpott MP, Green MR, Kealey T: Human hair growth *in vitro*. J Cell Sci 97: 463–471, 1990

Philpott MP, Sanders DA, Kealey T: Whole hair follicle culture. Dermatol Clin 14:595–607, 1996

Price VH, Menefee E, Strauss PC: Changes in hair weight and hair count in men with androgenetic alopecia, after application of 5% and 2% topical minoxidil, placebo, or no treatment. J Am Acad Dermatol 41:717–721, 1999

Randall VA, Sundberg JP, Philpott MP: Animal and *in vitro* models for the study of hair follicles. J Investig Dermatol Symp Proc 8:39–45, 2003

Roenigk HH: New topical agents for hair growth. Clin Dermatol 6:119–121, 1988

Rouzaire-Dubois B, Dubois JM: K^+ channel block-induced mammalian neuroblastoma cell swelling: A possible mechanism to influence proliferation. J Physiol 510:93–102, 1998

Sanders DA, Fiddes I, Thompson DM, Philpott MP, Westgate GE, Kealey T: In the absence of streptomycin, minoxidil potentiates the mitogenic effects of fetal calf serum, insulin-like growth factor 1, and platelet-derived growth factor on NIH 3T3 fibroblasts in a K^+ channel-dependent fashion. J Invest Dermatol 107:229–234, 1996

Sato T, Li Y, Saito T, Nakaya H: Minoxidil opens mitochondrial $K(_{ATP})$ channels and confers cardioprotection. Br J Pharmacol 141:360–366, 2004

Schop RN, Goldberg MT: Nongenotoxicity of minoxidil in murine hair follicles as determined by the nuclear aberration assay. Toxicol Appl Pharmacol 92:150–154, 1988

Schwanstecher M, Sieverding C, Dorschner H, *et al*: Potassium channel openers require ATP to bind to and act through sulfonylurea receptors. EMBO J 17:5529–35, 1998

Seino S, Miki T: Gene targeting approach to clarification of ion channel function: Studies of Kir6.x null mice. J Physiol 554:295–300, 2004

Sinclair R: Observations on the clinical features of hypertrichosis. In: Camacho FM, Randall VA, Price VH eds. Hair and it's Disorders: Biology, Pathology and Management. London: Martin-Dunitz, 2000; p 339–346

Takeuchi S, Wangemann P: Aminoglycoside antibiotics inhibit maxi-K^+ channel in single isolated cochlear efferent nerve terminals. Hear Res 67:13–19, 1993

Thomas DG, Brinklow BR, Loudon ASI, Randall VA: Seasonal effects of prolactin and triidothyronine on hair follicle growth in red deer (*Cervus elaphus*). In: Milne JA (ed). Recent Developments in Deer Biology. Aberdeen: Macaulay Land Use Research Institute, 1994

Thornton MJ, Thomas DG, Brinklow BR, Loudon ASI, Randall VA: Breeding season mane hair follicles from red deer (*Cervus elaphus*) stags are stimulated by testosterone in whole organ culture. In Milne JA (eds). Recent Developments in Deer Biology. Aberdeen: Macaulay Land Use Research Institute, 1994; p 225–226

Thornton MJ, Thomas DG, Jenner TJ, Brinklow BR, Loudon AS, Randall VA: Testosterone or IGF-1 stimulates hair growth in whole organ culture only in androgen-dependent red deer follicles. In: Van Neste D, Randall VA (eds). Hair Research for the Next Millenium. Proceedings of the 1st Tricontinental Meeting of Hair Research Societies, Brussels. Amsterdam: Elsevier Science BV, 1996; p 311–314

Trube G, Rorsman P, Ohno-Shosaku T: Opposite effects of tolbutamide and diazoxide on the ATP-dependent K^+ channel in mouse pancreatic beta-cells. Pflugers Arch 407:493–499, 1986

Uno H, Cappas A, Brigham P: Action of topical minoxidil in the bald stump-tailed macaque. J Am Acad Dermatol 16:657–668, 1987

Valdeolmillos M, Nadal A, Contreras D, Soria B: The relationship between glucose-induced K^+ATP channel closure and the rise in $[Ca^{2+}]_i$ in single mouse pancreatic beta-cells. J. Physiol 455:173–186, 1992

Waldon DJ, Kawabe TT, Baker CA, Johnson GA, Buhl AE: Enhanced *in vitro* hair growth at the air-liquid interface: Minoxidil preserves the root sheath in cultured whisker follicles. In Vitro Cell Dev Biol Anim 29A:555–561, 1993

Wester RC, Maibach HI, Gur RH, Novak E: Minoxidil stimulates cutaneous blood flow in human balding scalps. Pharmacodynamics measured by laser doppler velocimetry and photopause plethymography. J Invest Dermatol 82:515–517, 1984

Yunoki T, Teramoto N, Ito Y: Functional involvement of sulphonurea receptor (SUR) type 1 and 2B in the activity of pig urethera1 ATP-sensitive K^+ channels. Br J Pharmacol 139:652–660, 2003

Zins GR: The history of the development of minoxidil. Clin Dermatol 6:132–147, 1988